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Localization of fumarylacetoacetate fumaryl hydrolase in rat liver.

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LOCALIZATION OF FUMARYLACETOACETATE FUMARYL HYDROLASE IN RAT LIVER

BY

KIRIT S. DOSHI

A Thesis

Submitted to the Faculty of Graduate Studies through the Department of Chemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

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1977

ABSTRACT

The intracellular location of FAH has been demonstrated in rat liver tissue. Two fractionation procedures involving homogenization and differential centrifugation were adopted. One fractionation procedure isolated the nuclear fraction while the other gave the mitochondrial, microsomal and soluble phase fractions. FAH is exclusively localized in the soluble phase of the rat liver cells. FAH also showed a high RSA in the soluble phase fraction. Fractionation efficiency was checked by microscopic studies and by determining the distribution of a nuclear marker enzyme, NAD-pyrophosphorylase, mitochondrial marker enzyme, cytochrome oxidase, soluble phase enzyme, adenosine deaminase and microsomal marker enzyme, glucose-6-phosphatase. Per cent distribution of total protein in each fractionation procedure was also determined. In the second fractionation procedure, efficiency was also checked by determining the per cent recoveries of FAH activity after each step of centrifugation.

ACKNOWLEDGEMENTS

I would like to thank my research advisor Dr. D. E. Schmidt Jr. for his inspiring direction and patient encouragement during the entire course of this research work.

Also, I would like to express my appreciation to Dr. Paul Taylor (Department of Human Kinetics), Dr. R. J. Thibert and Dr. B. Zak (Wayne State University, School of Medicine) for their advice and Ron Ewing for his help. Finally, I would like to thank Ginger Cheng for the typing of this thesis.

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ABBREVIATIONS

ADH	alcohol dehydrogenase
ATP	adenosine triphosphate
EDTA	ethylenediaminetetraacetate
FAH	fumarylacetoacetate fumarylhydrolase
LDH	lactate dehydrogenase
NAD, NADH	nicotinamide adenine dinucleotide, oxidized and reduced forms
NADP, NADPH	nicotinamide adenine dinucleotide phosphate, oxidized and reduced forms
NMN	nicotinamide mononucleotide
pHPPA	p-hydroxyphenylpyruvic acid
PKU	phenylketonuria
RSA	relative specific activity
TAT	tyrosine aminotransaminase

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CHAPTER I

INTRODUCTION

Fumarylacetoacetate fumarylhydrolase (EC. 3.7.1.2) catalyses the cleavage of fumarylacetoacetate into fumarate and acetoacetate. This reaction occurs in the last step of the degradative pathway of phenylalanine and tyrosine (Figure 1).

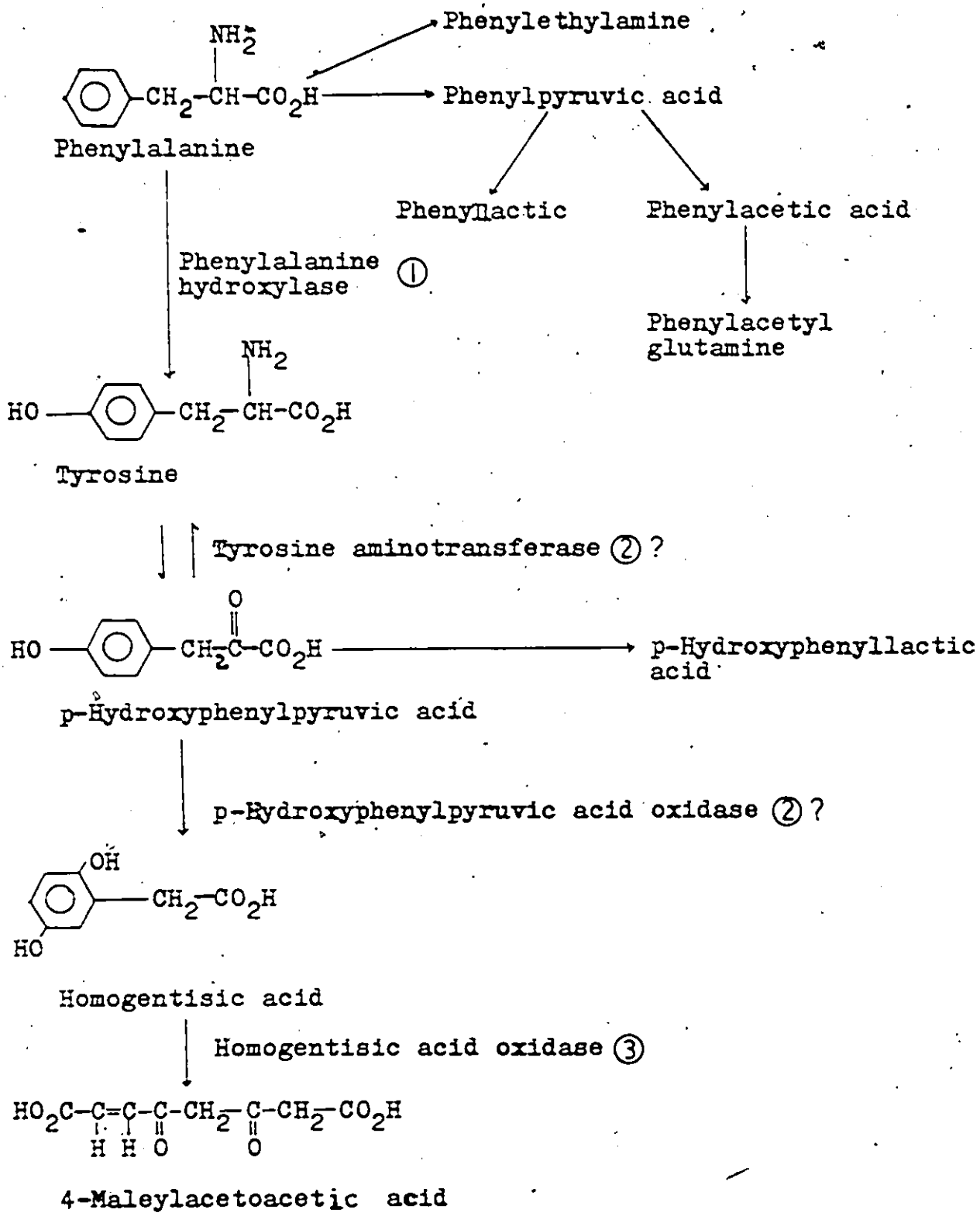
There are a number of hereditary diseases found in this pathway where the causal factor is lack of a particular enzyme. Of these, the most common one is phenylketonuria (PKU) where the enzyme phenylalanine hydroxylase is totally deficient. The biochemical and clinical abnormalities result from this deficiency.

Biochemically the disease is characterized by high levels of phenylalanine, phenylpyruvic acid (which is responsible for the mousy odour of the urine), phenyllactic acid and phenylacetylglutamine in the urine (1). Phenylalanine excretion is between 0.2 - 0.4 g per day as a result of an elevated serum level which saturates the renal transport mechanism. In adults, the level of phenylalanine in the serum reaches 15-60mg/100ml as compared to the normal level of about 1mg/100ml (1). cerebrospinal fluid also shows large amounts of phenylalanine. There is no increase in the serum concentration of phenylpyruvic acid and the other acid metabolites

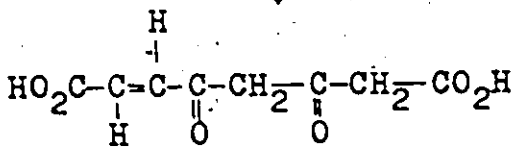
Figure 1. The Metabolism of Phenylalanine and Tyrosine. The irreversible conversion of phenylalanine to tyrosine is the major pathway. Minor routes are the formation of phenylethylamine and phenylpyruvic acid.

FIGURE 1

The Metabolism of Phenylalanine and Tyrosine

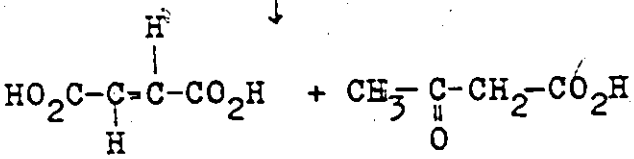


↓ Maleylacetoacetate isomerase



4-Fumarylacetoacetic acid

↓ Fumarylacetoacetate fumarylhydrolase



Fumaric acid Acetoacetic acid

① Deficient in phenylketonuria

② Deficient in tyrosinemia and tyrosinosis

③ Deficient in alkaptonuria

as the renal threshold for these acids is very low (1).

Clinically, the patients show fair hair and skin, marked mental retardation, hyperreflexia and some retardation of growth (1). The urine has a mousy odour. The disease is inherited as a Mendelian recessive and has an incidence of about 1:10,000. The condition of phenylketouric patients can be improved if they are maintained on diet containing very low levels of phenylalanine.

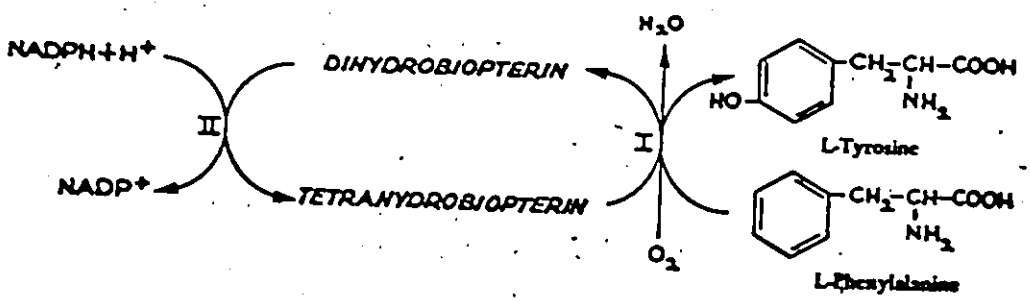
The enzyme, phenylalanine hydroxylase, is found only in the liver and is probably the regulatory enzyme for phenylalanine and tyrosine metabolism. It has been stated by Mahler and Cordes (2) that the enzyme is localized in the microsomal fraction of the cell. The enzymatic reaction involves two distinct enzymatic activities of which the functional component I of phenylalanine hydroxylase is absent in PKU (3). The component I converts phenylalanine to tyrosine with reduction of oxygen to water (Figure 2). The phenylalanine hydroxylase component II catalyses the reduction of dihydrobiopterin by NADPH.

The fact that the functional component I of phenylalanine hydroxylase (Enzyme I) is missing in PKU, was shown by Mitoma et al. (4), Wallace et al. (5) and Kaufmann (6).

Figure 2. The Phenylalanine Hydroxylase Reaction. (Reprinted from Harper (3) without permission of the publisher)

FIGURE 2

The Phenylalanine Hydroxylase Reaction.



Recently, Connellan (7) demonstrated two forms of phenylalanine hydroxylase in human liver obtained at autopsy. A soluble form remained in 6000 x g supernatant while a particulate form sedimented out at 6000 x g.

Barranger et al. (8) showed that phenylalanine hydroxylase exists as three distinct isozymes in adult rat which are chromatographically unique and differ only in charge. There are two isozymes existing in human fetal liver. By these isozymes they could explain much of the clinical variation of PKU. For example, genetic defects less extreme than that which causes complete loss of the enzyme might result from the absence or delayed maturation of one or more of the isozymes. This variation in genetic defects would produce varying degrees of total enzyme activity which might result in variable symptoms in the adult. Bessman (9) explained that if a fetal isozyme was absent or its appearance is delayed during rapid growth of development, brain growth and development would be impaired.

Tourian et al.(10) also demonstrated three immunologically distinct and noncrossreacting isozymes of rat liver phenylalanine hydroxylase distinguished by immunodiffusion, immunotitration and immunoabsorption. Miller and Shiman (11) showed that hydrocortisone stimulated phenylalanine hydroxylase isozymes in cultured hepatoma cells. Fisher and Kaufmann (12) showed

stimulation of rat phenylalanine hydroxylase by lysolecithin and α-chymotrypsin.

A second extremely rare disorder of tyrosine metabolism is tyrosinosis of which only a few cases have been thus far reported. The enzymatic defect in this syndrome is probably the absence of either hepatic p-hydroxyphenylpyruvate (pHPPA) oxidase activity or tyrosine aminotransaminase (TAT) activity. The patient with tyrosinosis excretes large quantities (1.5-3g/day) of tyrosine in the urine. On tyrosine-rich diet, other metabolites of tyrosine were also excreted.

Two cases of tyrosinosis have been reported; one by Lissitzsky et al. (13) and other by Fairney et al. (14). Fairney suggests that in his patient, tyrosinosis might be due to a deficiency of TAT in addition to the deficiency of pHPPA oxidase. This is supported by Hill and Zaleski (15) who found similar biochemical findings in their patient with tyrosinosis. However, both the patients of Fairney et al. (14) and Hill and Zaleski (15) show different clinical and biochemical symptoms from other cases of tyrosinosis.

Hereditary tyrosinemia is another disorder of the tyrosine catabolism and it was thought to be due to deficiency of the enzyme, pHPPA oxidase. This has been demonstrated in several cases to be the biochemical lesion (16,17). Affected patients show among other

symptoms, failure to thrive, nodular or adenomatous cirrhosis, renal proximal tubule dysfunction, edema and rickets. Biochemically the disease is characterized by massive excretion of pHPPA, p-hydroxyphenyllactic acid together with elevated blood and urine levels of tyrosine.

However, subsequent work shows that a deficiency of pHPPA oxidase may not be responsible for hereditary tyrosinemia. Bodegard et al. (18), who were among the first to propose pHPPA oxidase deficiency as the primary etiological factor in this disease suggest now that all the clinical findings of this disease cannot be attributed to be the result of pHPPA oxidase deficiency.

Fellman and his coworkers (19) found normal levels of pHPPA oxidase in a patient with tyrosinemia and of the two forms of TAT, the soluble form was virtually absent while the mitochondrial form was in normal amount. They also showed that hydrocortisone induced increased amounts of the enzyme both in mitochondrial and soluble fraction of rat liver.

Kennaway et al. (20) showed why the patient with tyrosinemia due to hepatic cytosol TAT deficiency and normal levels of pHPPA oxidase and mitochondrial TAT excretes pHPPA. Their recent studies on tissue distribution of these enzymes show that pHPPA oxidase activity is present only in human liver and kidney but is absent in muscle, heart and brain. Conversely mitochondrial TAT is present in all tissues (21). They conclude that

origin of pHPPA in the patient is from tissues which lack pHPPA oxidase.

Spencer and Gelehrter (22) induced the activity of TAT in hepatoma tissue culture cells with dexamethasone, insulin and serum. They stressed the need for caution in the interpretation of apparent multiple forms of enzymes since they detected cytosol aspartate aminotransferase, which has broad substrate specificity for aromatic amino acids. The aspartate aminotransferase acted as a "pseudoisozyme" of TAT. It has been shown that mitochondrial TAT is identical with mitochondrial aspartate aminotransferase (23). The synthesis of TAT also increases significantly with the administration of insulin or glucagon.

Williams and Sreenivasan (24) showed that the complete tyrosine oxidising system is found in the 25,000 x g supernatant and none in the precipitate. They prepared 16.7% rat liver homogenates (adult Sprague-Dawley rats) in 0.25M sucrose (isotonic) and centrifuged at 0°C for 1/2 hour at 25,000 x g. This procedure removed all particulate matter, including most of the microsomes and left the completely soluble enzymes in the supernatant solution. They then used this supernatant mixture of enzymes as the enzyme preparation in their subsequent experiments. They found that liver particulate fraction was totally devoid of the tyrosine oxidising system. From this indirect evidence, Fellman

and Vanbellingham in 1968 showed that the enzyme, pHPPA oxidase is found in the soluble phase but is absent in the mitochondria.

Kennaway and Buist (25) carried out metabolic studies in a patient with hepatic cytosol TAT deficiency but had normal levels of pHPPA oxidase (hydroxylase) and hepatic mitochondrial TAT.

Above results suggest the need to reexamine sub-cellular distribution of enzymes both in normal subjects and in patients with inborn errors of metabolism. The isoenzymes of TAT may be under individual genetic control. Accumulation of an intermediary metabolite should not be taken as conclusive evidence of a primary abnormality of its catabolism.

Chesnokov and Mertvetsov (26) fractionated the isoenzymes of rat liver TAT by DEAE cellulose chromatography, which showed two distinct fractions corresponding to the anodic and cathodic isoenzymes established by agar gel electrophoresis. Multiple forms of TAT have been resolved using polyacrylamide-gel-electrophoresis (27) and agar-gel-electrophoresis (28), hydroxyapatite chromatography (29,30) and ion exchange chromatography (31). According to all these workers TAT exists in at least three isozymic forms in rat liver. Iwasaki et al. (32) also showed three forms of TAT from rat liver and a fourth form from kidney, brain, heart and other tissues. Studies by Holt and

Oliver (27) and Iwasaki and Pitot (29) indicate that hormones which induce TAT might do so by separately inducing certain of these multiple forms. Recently Johnson et al. (31) have suggested that several hormones induce a single form of TAT and that this form can interconvert with other forms post-translationally.

Hereditary tyrosinemia can be treated in several cases by a synthetic diet, low in phenylalanine and tyrosine. This dietary treatment generally cures renal tubular lesions i.e. hyperphosphaturia, hyperaminoaciduria, glucosuria, proteinuria as well as rickets, acidosis and ascites.

Finally, the fourth, rare biochemical defect found in tyrosine metabolism is alkaptonuria in which deficient enzyme is homogentisic acid oxidase; this has been clearly demonstrated in both liver and kidney. In alkaptonuric patients all other enzymes involved in the tyrosine metabolism to acetoacetic acid are present and have about the same activity as in normal liver as shown by Table I (33,34). Homogentisic acid oxidase is found in the soluble fraction of liver and kidney (35). Alkaptonuria can occur only when there is a universal enzyme defect; no homogentisic acid oxidase was observed in liver and kidney in the affected patients (36).

Clinically, the disease is characterized by exessive amounts of homogentisic acid in the urine which darkens on exposure to air. The concentration of homogentisic

Table I. Activity of Tyrosine Oxidation Enzymes in Alkaptonuric and Nonalkaptonuric Human Liver Homogenate. (Reprinted from La Du *et al.* (33) without permission of the publisher)

Table I

Activity of Tyrosine Oxidation Enzymes in Alkaptonuric and Nonalkaptonuric Human Liver Homogenates (33)

Enzyme	Enzyme activity, μmoles of substrate oxidized/hr/gm of liver	
	Nonalkaptouric	Alkaptouric
Tyrosine transaminase	36	32
p-hydroxyphenylpyruvic acid oxidase	67	46
Homogentisic acid oxidase	268	<0.048
Maleylacetoacetic acid isomerase*	960	780
Fumarylacetoacetic acid hydrolase	288	222

*Units calculated as Δ log optical density per hr per 0.1 gm wet weight of liver (37)

acid in the plasma is approximately 1.5-3.5 mg/100ml. Alkaptonuria is inherited as an autosomal recessive character and in early life produces no clinical symptoms. In later life melanin-like pigment is deposited in cartilage and tendon. The ears and nose become blue-black in colour and arthritis is common feature in the late course of the disease.

Treatment of alkaptonuria is directed towards correcting the underlying metabolic defect or preventing or reversing the pigmentation and arthritic changes. Several vitamins, brewer's yeast, tyrosinase, insulin and adrenocortical extract have been administered without altering the amount of homogentisic acid excreted in urine by an alkaptonuric patient. Other agents, such as vitamin B_{12}, cortisone and phenylbutazone are without influence on the metabolic defect.

Both enzymes involved in the final steps of tyrosine metabolism, maleylacetoacetate isomerase and fumarylacetoacetate fumarylhydrolase (FAH) were first purified and separated from soluble fraction of rat liver (38). The purpose of this thesis is to determine the intracellular location of FAH in rat liver using technique of homogenization and differential centrifugation. These studies will show in which subcellular fraction the end of the tyrosine catabolism occurs and may indicate whether FAH has any isozymic forms and if so their cellular distribution.

CHAPTER II

EXPERIMENTAL

A. METHODS AND MATERIALS

1) Materials

The following materials were commercially available, sucrose, cytochrome c, adenosine, glycylglycine buffer, ATP, NMN, nicotinamide, phenol red, alcohol dehydrogenase (crystalline) (Sigma Chemical Co.); NaCl, $CaCl_2$, potassium phosphate, potassium ferricyanide, KOH, $MgCl_2$, TCA, $Na_2S_2O_4$ (Fisher Scientific Co. Ltd.); 95% ethanol (Consolidated Alcohols Ltd.); disodium EDTA, $NaH_2PO_4 \cdot H_2O$ (J. T. Baker Chemical Co.); citric acid (Mallinckrodt Chemical Works)

2) Enzyme Location in Rat Liver

Rats were obtained from either the Department of Psychology or the Department of Biology, University of Windsor. The experiments were performed on the livers of adult rats kept on standard diet. In all cases, the animals were fasted for at least 12 hr before death. Two fractionation procedures were adopted. Nuclei were isolated on a small scale according to a method used by Dounce (39) with some modifications as described below. Mitochondria, microsomes and soluble phase were fractionated by a method used by de Duve *et al.* (40). All steps in the fractionation procedures were carried out at 4°C. All equipment, glassware and solutions

were thoroughly chilled at 4°C before use.

(a) Nuclei

Step 1

One rat was stunned and decapitated. The liver was quickly extracted and was perfused first with cold isotonic saline, to remove the erythrocytes, and then with 0.25 M sucrose containing 0.002 M $CaCl_2$. The use of calcium ions is desirable in preventing swelling and gelation of the nuclei. A 7 g portion of the washed liver was placed in a Waring Blender and homogenized for 45-50 sec with 60 ml of 0.25 M sucrose containing 0.002 M $CaCl_2$. Excessive homogenization was strictly avoided since it tends to damage the nuclei and lowers the yield. The crude homogenate was filtered through four layers of cheesecloth to remove any connective tissue. The filtered homogenate was thoroughly mixed and examined under 50 fold magnification with a Bausch and Lomb microscope. Further homogenization with a teflon homogenizer was not carried out since no whole cells were seen in the homogenate. Sixteen milliliters of the homogenate were removed and stored for future analysis.

Step 2

A 25 ml portion of the filtered suspension was placed into each of two 50 ml steel centrifuge tubes and each was underlaid with 25 ml of 0.34 M sucrose containing 0.0002 M $CaCl_2$ with the aid of a pipet. Care was taken so as not to disturb the phase boundary. The fractions

were centrifuged at 2900 rpm (1000 x g) for 10 min at 3°C in a Sorvall RC2-B refrigerated centrifuge. The tubes were carefully removed from the centrifuge and the supernatant fractions were decanted into a 100ml beaker; this is referred to as the SI fraction.

Step 3

The two loosely packed nuclear pellets referred as N were each suspended in 25 ml of 0.25 M sucrose (without $CaCl_2$) and pooled. Using a Radiometer PH Meter 26, the suspension was adjusted to pH 6.2 by careful addition of 0.01 M citric acid. Nuclei are more stable at acidic pH and a generally low pH gives higher purity of the nuclear fraction. However, for enzyme work a pH of 6-6.2 is more desirable (41). The citrate ions without further addition of $CaCl_2$ prevents clumping of nuclei and mitochondria (39) and also inhibit the activity of the "neutral" deoxyribonucleases. The 25 ml portions were then placed into 50 ml centrifuge tubes and underlaid, as before with 25 ml of 0.34 M sucrose (without $CaCl_2$). The fraction was centrifuged at 1800 rpm for 10 min at 3°C. The supernatant fractions were poured into a 100ml beaker as before and termed the SII fraction.

The treatment as from beginning of step 3 was repeated four more times; however no pH adjustments were necessary in these washes. The resultant nuclear fraction had very small amount of mitochondria and other subcellular particles. The supernatants of these washes were termed

SIII, SIV, SV and SVI fractions. The final nuclear pellet was centrifuged at 1200 rpm for 10 min at 3°C. The washed nuclear pellet was suspended in 15 ml of 0.25 M sucrose solution.

Widnell et al. (42) had presented a method for nuclei isolation. This method was not used because the nuclear fraction gave low yields with marked cellular contamination; the latter may be due to incomplete homogenization. The method used here gave a good yield of nuclei with very little whole cell and partially broken cell contamination.

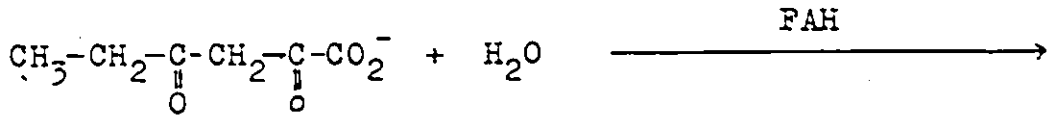
Analysis of the fractions was done as soon as possible. Microscopic examination of all fractions including the homogenate were done using a Bausch and Lomb microscope. The fractions were divided into two sets. One set of the fractions was rapidly frozen in liquid nitrogen and then thawed by swirling the tubes in warm water; this was repeated twice more. The freeze and thaw method was used to disrupt the thus far unbroken cell organelles and to expose FAH and other latent enzymes, like cytochrome oxidase, involved in the assay procedures shown below. The second, undisrupted set of fractions was refrigerated until needed for the NAD-pyrophosphorylase assay. The undisrupted fractions were used for this assay because the freeze and thaw procedure might affect the low activity of NAD-pyrophosphorylase.

(1) Determination of FAH Activity:

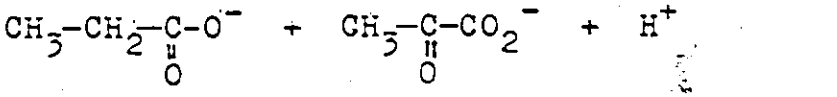
Standard Assay:

An artificial substrate, propionopyruvate is used to

determine the activity of FAH. Propionopyruvate was found to be most reactive with FAH of a number of diketo acids studied (40). The enol form of propionopyruvate absorbs at 290 nm. FAH catalyses the cleavage of propionopyruvate to give propionate and pyruvate, resulting in a decrease of absorption at 290 nm.



Propionopyruvate



Propionate Pyruvate

The standard reaction mixture consists of 2.6 ml of 0.025 M sodium phosphate buffer, pH 7.3 and 0.3 ml of 0.2 mM propionopyruvate. The reaction was initiated by addition of 0.05 ml or 0.1 ml of the disrupted fraction and decrease in absorption at 290 nm was followed for about 5 min on a Gilford Model 2000 Absorbance Recorder attached to a Beckman DU monochromator. The initial rate was determined from a slope of a tangent drawn at 1/2 min of the absorption trace. The controls were also run for each fraction treating them similarly except the substrate and these "background" absorptions were subtracted from the above set of absorptions to give the net decrease in absorptions. The value of 4.81ml/ µmoles.cm as molar extinction coefficient (ε) for the propionopyruvate is utilized (43).

Determination of activity of FAH in terms of μmoles of propionopyruvate hydrolysed/min.:

One unit of enzyme is defined as the amount of enzyme that hydrolyses 1 μmole of propionopyruvate/min

$$= \frac{\Delta O.D./min}{\varepsilon \times 1} \times (\text{dilution factor}) \times (\text{total vol of the fraction})$$

where 1 = path length of the cell = 1.0 cm

The specific activity,

$$S.A. = \frac{\text{units of FAH}}{\text{total protein in mg}} = \text{units/mg of protein}$$

(2) Determination of Total Protein:

Total protein was determined using the Beckman spectrophotometer by the method of Warburg and Christian (44). Absorbance of all fractions were read at 260 and 280 nm after suitable dilutions. Protein in mg/ml were calculated from the nomograph and hence the total protein in mg could be calculated in the whole fraction.

(3) Marker Enzymes:

One of the best ways to check the efficiency of the fractionation procedure is by the use of marker enzymes. Many marker enzymes are commonly used for such control experiments. A marker enzyme is an enzyme which is known to be exclusively localized in a particular component of the cell and by checking each of the fractions of the preparation for the amount of enzyme activity that each contains, the degree of efficiency in the fractionation

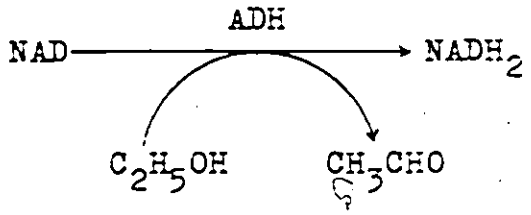
can be established. For nuclear fractionation procedure, three such marker enzymes were used. The first one was NAD-pyrophosphorylase (Relative Specific Activity, RSA = 5.5), an exclusively nuclear enzyme; the second marker enzyme was cytochrome oxidase (RSA = 3.1), an exclusively mitochondrial enzyme; and the third one was adenosine deaminase (RSA = 2.7), an exclusively soluble phase enzyme (45).

(a) NAD-Pyrophosphorylase

NAD-pyrophosphorylase catalyses the reaction:

$$ATP + NMN \rightleftharpoons NAD + PP_i$$

NAD synthesized by the enzyme is reduced, in presence of enzyme alcohol dehydrogenase (ADH) and ethanol, to $NADH_2$ which has an absorption at 340 nm; hence an increase in absorption at 340 nm could be monitored.



The assay procedure of NAD-pyrophosphorylase was closely followed according to the one used by Hogeboom and Schneider (46).

The reaction mixture (pH7.5) was made up at 0°C; 0.9 ml of the mixture contained: 0.006 M ATP, 0.0025 M NMN, 0.05 M glycylglycine - KOH buffer, 0.015 M $MgCl_2$,

0.2 M nicotinamide and 0.1 ml of enzyme fraction. The use of nicotinamide in these assay fractions avoids interference by NAD nucleosidase (47,48). A control is treated the same except no enzyme fraction was added. The assay tubes were incubated at 38°C in Forma-Temp bath for 5 min before the addition of the enzyme fraction. The reaction was allowed to occur for 20 min at 38°C after which it was stopped by addition of 1.0 ml of cold 10% TCA. The tubes were centrifuged at 0°C and the supernatants were carefully neutralized while cold with 2.4 N KOH with phenol red as an internal indicator. The color change was from yellow (acidic) to just pink (neutral). A 1.0 ml aliquot was then placed in 1 ml, 1 cm path length cuvette, together with 0.3 ml of 0.1 M sodium pyrophosphate, pH 9.0 and 0.1 ml of a solution of crystalline alcohol dehydrogenase. Then 0.1 ml of ethanol was added to it and the absorption at 340 nm was followed for at least 20 min. The rate of synthesis of NAD in fractions which had NAD-pyrophosphorylase, proceeded linearly with time for 20 min. The control which was treated exactly same except 0.1 ml of water instead of 0.1 ml ethanol was added, showed no formation of NAD. The value of 6.27 $cm^2/\mu mole$ as molecular extinction coefficient, ε, for the reduced NAD was utilized (49).

Determination of activity of NAD-pyrophosphorylase in terms

of μmoles of NAD synthesized/hr:

$$\mu\text{moles of NAD syn./hr (units)} = \frac{\text{O.D./min}}{\epsilon \times 1} \times$$

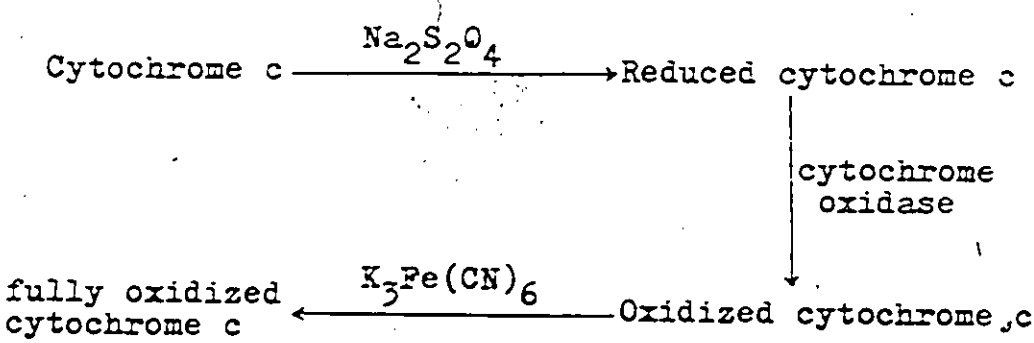
(Total vol of NAD soln in assay (3 ml)) x

$$\frac{\text{Vol. of fraction}}{\text{Vol. of enzyme aliquot used}} \times 60 \text{ min}$$

where 1 = path length of the cuvette (1 cm)

(b) Cytochrome Oxidase:

Cytochrome oxidase catalyses reduced cytochrome c to its oxidized form. Prior to the assay the cytochrome c is reduced with enough solid sodium hydrosulfite, $Na_2S_2O_4$. The reduced form of cytochrome c has the absorption at 550 nm in the visible region; hence the decrease in absorption could be followed for 3-6 min. Then the blank could be read off after addition of solid potassium ferricyanide which fully oxidizes the cytochrome c.



The assay for cytochrome oxidase was carried out according to Applemans et al. (50). The fractions were not diluted in 0.005 M potassium phosphate buffer, pH 7.4 as the dilution gave low activities. A 1.7×10^{-5} M freshly prepared solution of cytochrome c in 0.03 M potassium phosphate buffer, pH 7.4 was reduced by adding a small amount of solid $Na_2S_2O_4$ to give approximately 90% reduced cytochrome c. A 3 ml aliquot of substrate was placed in a cuvette and the reaction was started with the addition of 0.05 ml of the enzyme fraction. In case of homogenate and SI fraction 0.01 ml aliquots were taken since the "background" absorptions of the controls were high when 0.05 ml aliquots were used. The decrease in absorption at 550 nm was measured every minute for 4 min, after which the blank was read following addition of a small amount of solid potassium ferricyanide.

In all cases absorption of the controls were followed using 3 ml of 0.03 M potassium phosphate buffer instead of the substrate, cytochrome c. The "background" absorptions were subtrated from the first set of absorptions to give the actual decrease in absorption caused by cytochrome oxidase.

Determination of activity of cytochrome oxidase:

One unit of activity of cytochrome oxidase is defined as the amount of enzyme causing the decadic logarithm of the concentration of reduced cytochrome c to decrease by one unit/min/100 ml of incubation mixture. The calculations

are carried out according to Cooperstein and Lazarow (51).

$$\Delta \log_{10}(\text{ferrochrome c}) / \Delta t = \frac{\log_{10}(O.D._{t_1} - O.D._{ox}) - \log_{10}(O.D._{t_2} - O.D._{ox})}{t_1 - t_2} \times$$

$$(\text{dilution factor}) \times (\text{vol of fraction}) \times \frac{1}{100}$$

Where $O.D._{t_1}$, $O.D._{t_2}$ and $O.D._{ox}$ are the extinctions at times t_1, t_2 and after addition of ferricyanide respectively.

(c) Adenosine Deaminase:

This enzyme catalyses the deamination of adenosine to inosine.

$$\text{Adenosine} + H_2O \xrightarrow[\text{Deaminase}]{\text{Adenosine}} \text{Inosine} + NH_3$$

At 265 nm, adenosine has a larger molar extinction coefficient than inosine while at 240 nm the reverse is true. However, since the magnitude and hence the accuracy of absorption at 265 nm is twice as great as at 240 nm, absorptions were read at 265 nm. The assay procedure was followed at room temperature according to Schneider and Hogeboom (52).

The components of the reaction mixture were: 0.6 ml of 0.25 M glycylglycine buffer, pH 7.4, 0.05 ml of the enzyme fraction and 2.4 ml of deionized water. After

equilibrium at room temperature, 0.1 ml of 0.002 M adenosine, freshly prepared, was added and readings were taken at 0, 10 and 20 min at 265 nm on the Beckman spectrophotometer.

Determination of adenosine deaminase activity in terms of micromoles of adenosine deaminated/min:

$$\mu\text{moles of adenosine deaminated/min (units)} = \frac{\text{O.D./min}}{\epsilon \times 1} \times \text{(dilution factor)} \times \text{(volume of the fraction in lit)}$$

Where ϵ is the molar extinction coefficient of adenosine of the value, 1.51×10^{-2} lit/μmoles·cm as reported by Kalckar (53).

and 1 is the path length of the cuvette = 1 cm.

The enzyme, lactate dehydrogenase (LDH) was not used as a soluble phase marker for two reasons. First, several fractions showed large absorption at 340 nm even when no substrate was present; these absorptions falsely showed the presence of LDH activities in fractions where they were not expected. Secondly, the "background" absorptions of these control assays completely masked the absorptions due to LDH activities, if any, present in the assay fractions.

(b) Mitochondria, Microsomes and Soluble Phase

Step 1

One adult rat was stunned and decapitated. The liver was quickly extracted and perfused twice in medium of 0.25 M

sucrose containing 0.001 M disodium EDTA. EDTA acts as a chelating agent to bind any calcium ions (54) which even in small amounts would inhibit oxidative phosphorylation in the mitochondria (55). Calcium ions also bring about mitochondrial swelling (56). EDTA exerts a protective action on glucose-6-phosphatase (57) and probably on other enzymes (58).

A 10 gm portion of the liver was taken and minced into 2 - 3 mm pieces with a razor. The minced tissue was placed in a homogenizer of Potter and Elvehjem type, consisting of a smooth walled glass tube fitted with a Teflon pestle, and 30 ml of the medium was added to it. The tube was given a single run upward against the rapidly rotating pestle at 1500 rpm until all the material had been forced above the latter. The resulting slurry was centrifuged using Sorvall RC2-B refrigerated centrifuge at 3°C at 10,000 g-min. (3,000 rpm for 13½ mins). The supernatant was poured into a 100 ml beaker. The sediment which still contained a large number of unbroken cells in addition to nuclei, was rehomogenized as before in 30 ml of the medium and centrifuged at 3°C at 6,000 g-min. (3,000 rpm for 8 min). The repetition of this operation gave the nuclear fraction, referred to as the N fraction, almost free of intact cells or gross debris. The N fraction was redispersed in 40 ml of the medium, giving a 1 : 4 nuclear fraction. This supernatant was combined with the first one and the whole was made up to

110 ml with the medium; this was termed as fraction, E. A 10 ml portion was removed and stored for future analysis.

The sum of the values obtained separately on the nuclei, N and cytoplasmic extract, E was taken as representative of the whole tissue i.e. the homogenate, since mixtures of these two fractions usually undergo extensive agglutination and sometimes yield erratic results (40).

Step 2

The E fraction was further centrifuged in 40 ml portions at 33,000 g-min. (6,000 rpm for 11 min) at 3°C. The sediments were collected in one tube, resuspended in 10 ml of medium using the homogenizer and recentrifuged at 6,000 rpm for 11 min. This was repeated one more time. The washed pellet was finally suspended in about 20 ml of medium to give 1 : 2 heavy mitochondrial fraction, M. The supernatants from the above, including the two washes were combined and referred to as fraction F; 5 ml of this was removed and stored for future analysis.

The rest of the fraction F was centrifuged at 250,000 g-min. (16,000 rpm for 12 min) at 3°C. The pellets were washed twice as before and finally suspended in about 20 ml of the medium to give 1 : 2 light mitochondrial fraction, L. The three supernatants were pooled as before and termed fraction G; 5 ml of this was removed and stored for future analysis.

Fraction G was centrifuged at 3,000,000 g-min. (35,000 rpm for 33 min) at 3°C using the International Preparative Ultracentrifuge Model B-60. The pellets were suspended in 25 ml of the medium and referred as microsomal fraction, P. The final supernatant was the soluble phase fraction, S.

The heavy mitochondrial sediment consisted of a reasonably well-packed bottom layer of a homogenous light brown color. The light mitochondrial sediment occurred in the form of a dark brown pellet while the microsomal pellet was detected as a dark red jelly-like pellet. After each centrifugation, a white fluffy layer was seen floating at the top of the supernatant. This was removed in each case.

The above method for separating particulate fractions used the nomenclature of g-min. to ensure reproducibility when other types of centrifuges are used by other workers (50). This is useful since different types of instruments cannot be expected ever to furnish identical results. This fact may be partly responsible for the wide variations which are sometimes encountered between results obtained in different laboratories (59). The nomenclature of g-min. is fully explained and justified by Duve and Berthet (59) and Applemans et al. (50). The calculations for g-min. are shown in the Appendix.

All fractions were then examined under 100 x oil immersion using a Bausch and Lomb microscope as before. Following this the fractions were disrupted

using the freeze and thaw method as before to expose the latent enzymes from the thus far unbroken cell organelles. The fractions were stored under ice in the cold room until required for the enzyme assays. Except for glucose-6-phosphatase the enzyme assays stated below were performed as described previously.

(1) Determination of FAH activity;

(2) Determination of Total Protein;

(3) Marker Enzymes:

(i) Cytochrome oxidase;

(ii) Adenosine deaminase;

(iii) Glucose-6-phosphatase.

For mitochondrial, microsomal and soluble phase fractionation, glucose-6-phosphatase is selected as a microsomal marker enzyme since it is exclusively associated with this cell organelle (RSA = 3.0), (45).

Glucose-6-Phosphatase:

Glucose-6-phosphatase (G6Pase) catalyses the reaction:

$$\text{Glucose-6-phosphate} + H_2O \longrightarrow \text{Glucose} + P_i$$

The amount of inorganic phosphate (P_i) liberated could be determined by the most widely used method of Fiske and SubbaRow (60). P_i reacts with ammonium molybdate in an acid solution to form phosphomolybdic acid. Fiske & SubbaRow reducer is added which reduces the molybdenum to give a blue color but does not affect the uncombined molybdic acid. The absorbance of the blue color can be measured at 660 nm.

The assay procedure of G6Pase was partly followed according to De Duve et al. (40).

One ml reaction mixture (pH 6.5) contained 0.04 M glucose-6-phosphate, 0.007 M histidine, 0.001 M EDTA and 1.4×10^{-3} M $BeSO_4$. The use of $BeSO_4$ was to inhibit alkaline phosphatase activity (61) that may interfere with the G6Pase activity. The mixture was incubated at 37° for 10 min after which 0.3 ml aliquot of the fraction was added. The reaction was stopped after 20 min by the addition of 10% (w/v) trichloroacetic acid. The precipitated protein was centrifuged and 1 ml of the clear supernatant was removed for the determination of the P_i.

Determination of inorganic phosphate:

To 1 ml of the clear supernatant, 2 ml of deionized water, 0.5 ml of 2.5% w/v ammonium molybdate in 5 N H_2SO_4 (62) and 0.2 ml of Fiske & SubbaRow reducer were added. The tubes were well stirred on each addition of the reagents. The blue color was allowed to develop for 20 min after which absorbance was read off at 660 nm from the Beckman spectrophotometer (62).

In the second fractionation procedure the N, M and L fractions were contaminated with substantial amounts of microsomes which may have become closely associated with the nuclei and mitochondria. In order to reduce the contamination of microsomes each of the three fractions were washed at least five times. The nuclear pellet was suspended using the glass rod. In case of M and L fractions, the pellets were

suspended with the four up and down movements of the Teflon homogenizer. This treatment removed the majority of the microsomes from these fractions. The supernatants of the N, M and L fractions were combined with E, F and G fractions, respectively.

Prior to G6Pase assay, each fraction was immersed in ice and homogenized with four up and down movements of the Teflon homogenizer to give a smooth suspension (63). The fractions were then subdivided into two portions and frozen until required for the assay.

Controls for the G6Pase assay were also carried out by incubating 0.3 ml of each fraction in 1.0 ml of 0.1 M Bis Tris buffer, pH 5.00 at 37° for 20 min. This treatment would destroy the G6Pase (61). The assay was then carried out as before on the G6Pase killed system. This treatment is said to account for nonspecific enzyme activity stable under the conditions in which G6Pase is labile (63). These absorbances at 660 nm were subtracted from the first set of absorbances to give the true G6Pase activity. In addition, chemical inhibition of G6Pase is a well-practised phenomenon (61).

Determination of G6Pase activity:

The activity in units is calculated on the basis of the amount of inorganic phosphate (Pi) liberated where a unit equals one μmole of Pi released per minute.

Initially units/ml of Pi supernatant can be calculated from the standard curve of Pi determination.

Standard curve of Pi determination:

Standard solution of Pi prepared by dissolving 4.0839 g of analytically pure KH_2PO_4 in 1000 ml of deionized water and a few drops of chloroform is added (64). For use, it was diluted 1:10 so that 1 ml corresponds to 3 µmoles of phosphorus.

One ml standards contained between 0.5 to 3 µmoles of phosphate. To this, 2 ml of deionized water, 0.5 ml of 2.5% ammonium molybdate in 5 N H_2SO_4 and 0.2 ml of Fiske & SubbaRow reducer were added and absorbances were read off after 20 min as before.

From the standard curve units of G6Pase/ml of the supernatant can be calculated. Units of G6Pase can be calculated as follows:

$$\text{Units of G6Pase} = \frac{\text{units/ml}}{20 \text{ min}} \times (\text{Vol. of Pi supernatant (2.3 ml)}) \times \frac{(\text{Vol. of fraction})}{(\text{Vol. of enzyme aliquot used})}$$

B. RESULTS

Enzyme localization in Rat Liver

(a) Nuclei

The nuclei were isolated and the flow sheet showing the steps of the fractionation procedure is shown in Figure 3. Microscopic studies were in agreement with the implications shown in this flow sheet of the fractionation. After centrifugation at 1000 x g (3,000 rpm), the SI supernatant contained most of the mitochondria, microsomes and the soluble phase. The NI precipitate contained nuclei, some mitochondria and a small amount of cell debris. This crude NI precipitate underwent five successive washes giving a highly purified fraction of nuclei, N. This procedure removed almost all of the contaminating mitochondria, microsomes and soluble phase from the N fraction. Fractions SII to SVI all contained mitochondria, microsomes and soluble phase but appeared in increasing dilution towards SVI under the microscopic examination. The N fraction appeared dense with nuclei distinctly visible. Some nuclei were present in clumps. Nuclei isolated by the method used here (39) were in good yield, undamaged and in pure state as shown by the marker enzyme activities discussed later.

Table II shows the volume, total protein, total FAH activity and specific activity of all the fractions for a single fractionation of rat liver. The Table indicates that SI fraction contains 7.56 units of FAH activity as

Figure 3. Flow Sheet showing the Fractionation of Nuclei in Rat Liver. The details of each step can be found in the EXPERIMENTAL section of this chapter. The components of each fraction are indicated as obtained from microscopic examination.

Figure 3

Flow Sheet showing the Fractionation of Nuclei in Rat Liver

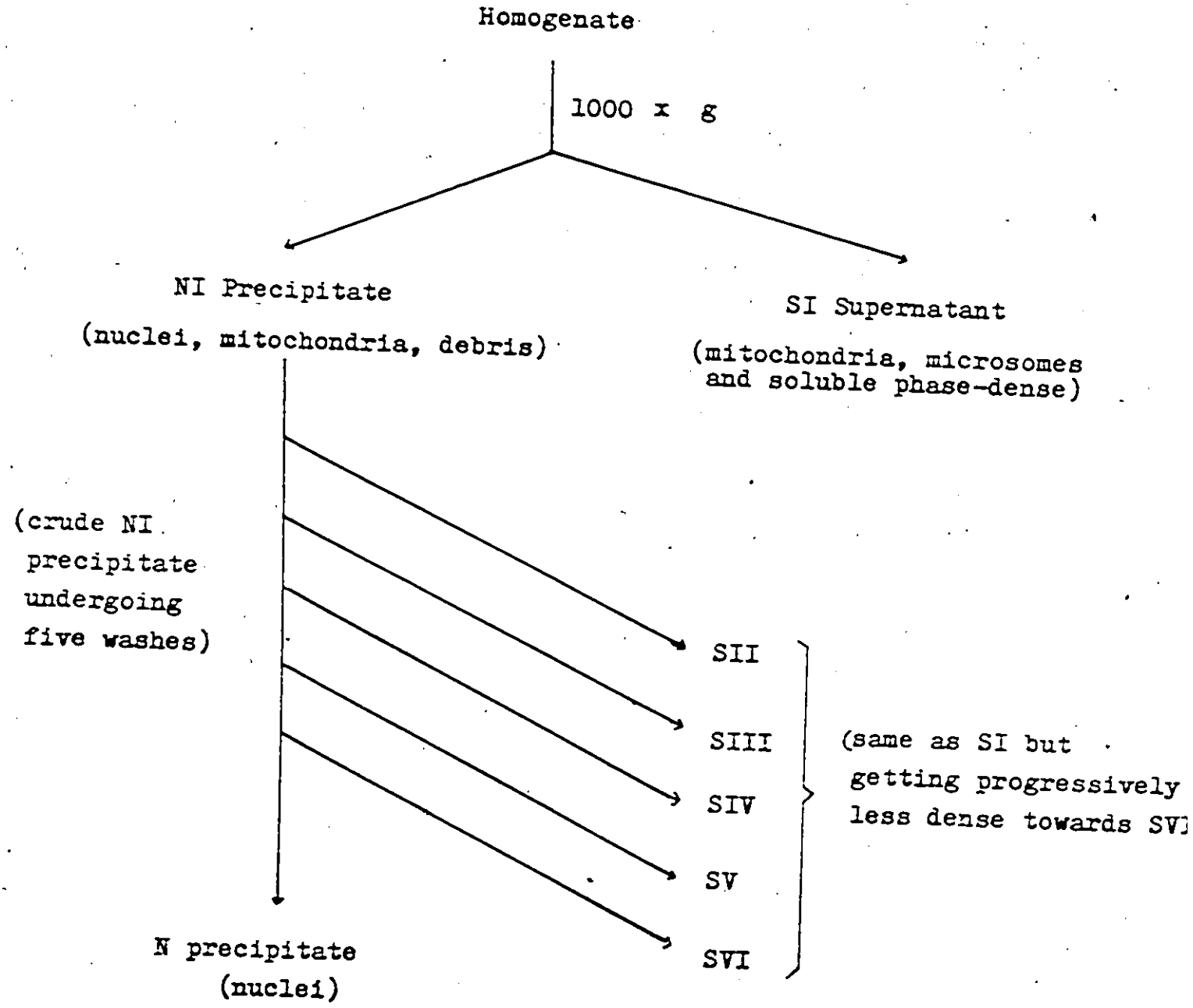


Table II. PAH Localization in Rat Liver Fractions: Nuclei. The details of the assay can be found in the EXPERIMENTAL section of this chapter. Protein was determined spectrophotometrically by the method of Warburg and Christian (44).

Table II

FAH Localization in Rat Liver Fractions : Nuclei

Fraction	Volume ml	Total Protein mg	Total FAH activity in units*	Specific Activity units/mg x 100
Homogenate	50	3660	5.95	0.16
SI	80	2352	7.56	0.32
SII	62	1097	0.46	0.04
SIII	43	290	0.27	0.09
SIV	40	65	0.50	0.77
SV	40	20	0.50	2.50
SVI	34	5	0.49	9.80
N	15	77	0.35	0.45

*Micromoles of propionopyruvate hydrolysed/min.

compared to 0.35 unit in the N fraction. This means that the SI fraction retained about 21 times more FAH activity than the N fraction. Supernatants SII to SVI contained between 0.27 to 0.50 units of FAH activities. This indicates that as the mitochondria, microsomes and soluble phase were undergoing separation, FAH activity associated with these cell organelles and soluble phase was also detected. Figure 4 illustrates the per cent distribution of total protein in the rat liver fractions obtained from the nuclear fractionation. It shows 64.3% and 30% of the total protein of the homogenate was retained in the SI and SII fractions, respectively. The N and other supernatants contained smaller amounts of the total protein. The total recovery of the protein was 106.7% with deviation from 100% of 6.7% which is within experimental error. Since the homogenate of the nuclear fractionation represented low FAH activity, per cent distribution of the same was not calculated.

Marker Enzymes:

(i) NAD-Pyrophosphorylase:

Table III shows the determination and per cent distribution of NAD-pyrophosphorylase in rat liver fractions obtained from the nuclear fractionation. NAD-pyrophosphorylase, an exclusively nuclear enzyme should only be present in those fractions containing nuclei. The amount of NAD-pyrophosphorylase activity detected seems consistent with the values demonstrated by Hogeboom and Schneider (46).

Figure 4. Per Cent Distribution of Total Protein in Rat Liver Fractions: Nuclei. Letting the protein content of the homogenate be 100 per cent, the percentage of protein in other fractions is shown. The total should have been 100 per cent.

Figure 4

Per Cent Distribution of Total Protein

in Rat Liver Fractions : Nuclei

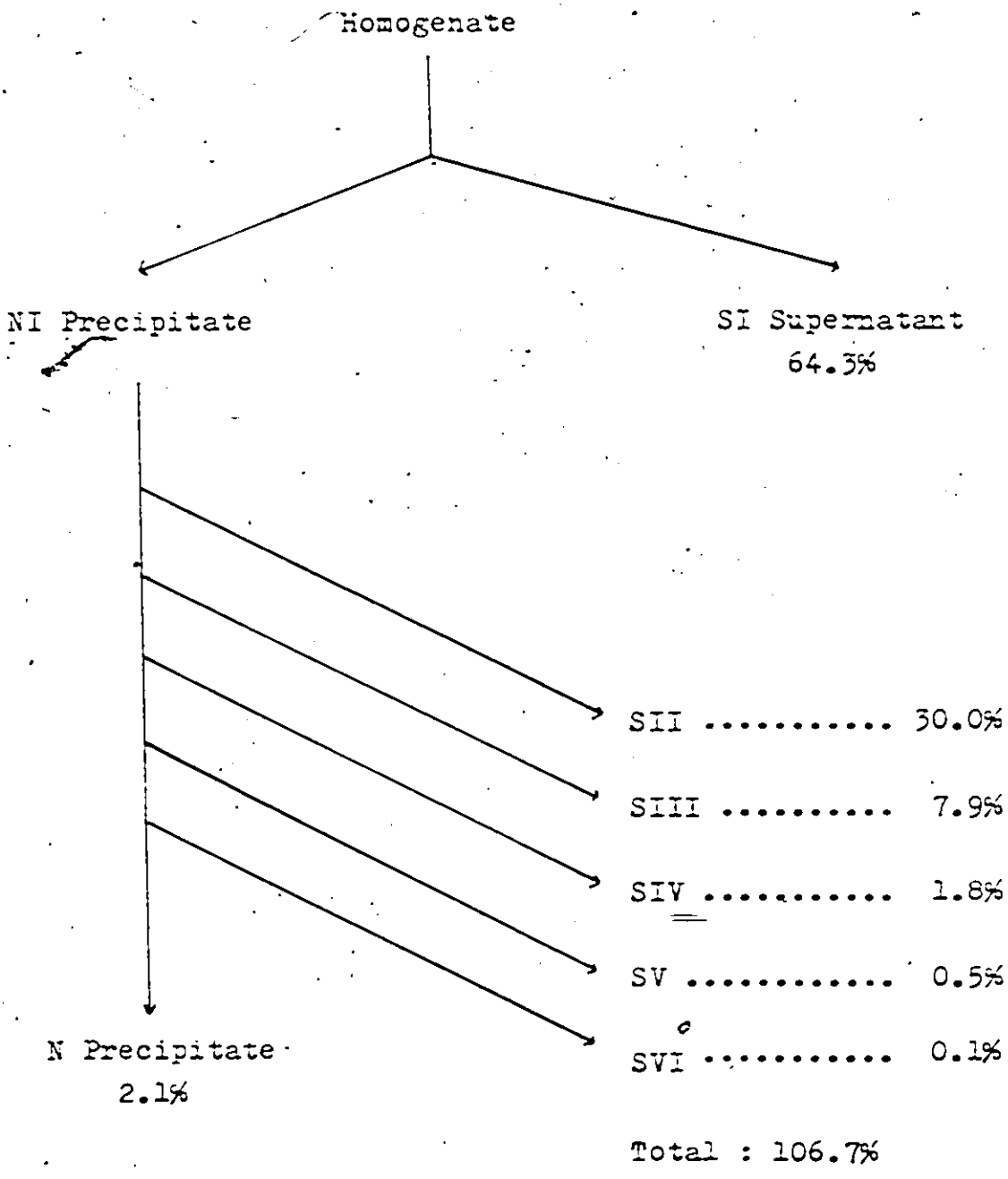


Table III. Determination and Per Cent Distribution of NAD-Pyrophosphorylase in Rat Liver Fractions: Nuclei. The details of the assay can be found in the EXPERIMENTAL section of this chapter. Letting NAD-pyrophosphorylase of the homogenate be 100 per cent, the percentage of NAD-pyrophosphorylase in other fractions is shown. The total recovery should have been 100 per cent.

Table III

Determination and Per Cent Distribution of NAD-Pyrophosphorylase in Rat Liver fractions : Nuclei

Fraction	Vol. in mls	NAD-pyrophosphorylase in units*	Per cent
Homogenate	50	1.67	100
SI	80	0	0
SII	62	0	0
SIII	43	0	0
SIV	40	0	0
SV	40	0	0
SVI	34	0	0
N	15	1.37	82

*expressed in micromoles of NAD formed per hour.

Total recovery of NAD-pyrophosphorylase = 82.0%

They recovered 0.78 unit of NAD-pyrophosphorylase in the nuclear fraction of 1.10 units found in the homogenate (46). The results of Table III shows that the N fraction retained 82% of NAD-pyrophosphorylase activity while all six supernatants, SI to SVI showed no activity. These results show that these six supernatants were not contaminated with nuclei and the N fraction contain all or most of the nuclei. The total recovery of NAD-pyrophosphorylase was 82.0% which is similar to the recovery of 92% obtained by Hogeboom and Schneider (46).

(ii) Cytochrome Oxidase:

Table IV shows the determination and per cent distribution of cytochrome oxidase in rat liver fractions obtained from the nuclear fractionation. Cytochrome oxidase which is exclusively mitochondrial enzyme, should only be found in fractions containing mitochondria.

From the results shown in Table IV, the SI and SII supernatants contained the majority of the cytochrome oxidase activity (32.2 and 21.1%, respectively). This indicates that the SI and SII fractions contained most of the mitochondria as expected. The supernatants SIII to SVI represented between 0.3 to 3.1% of the cytochrome oxidase activity showing the presence of some mitochondria which were separated from the N fraction. The N fraction showed the least amount of cytochrome oxidase activity (0.1 unit) indicating that the N fraction had extremely low

Table IV. Determination and Per Cent Distribution of Cytochrome Oxidase in Rat Liver Fractions: Nuclei. The details of the assay can be found in the EXPERIMENTAL section of this chapter. Letting cytochrome oxidase of the homogenate be 100 per cent, the percentages of cytochrome oxidase in other fractions are shown. The total recovery should have been 100 per cent.

Table IV

Determination and Per Cent Distribution of Cytochrome Oxidase in Rat Liver Fractions : Nuclei

Fraction	Vol in ml	Cytochrome Oxidase in units*	Per Cent
Homogenate	50	36.0	100
SI	80	11.6	32.2
SII	62	7.6	21.1
SIII	43	1.1	3.1
SIV	40	0.2	0.6
SV	40	0.1	0.3
SVI	34	0.1	0.3
N	15	0.04	0.1

*$\Delta \log_{10}$ [ferrochrome c] per min for 1 : 100 tissue dilution

Total recovery of cytochrome oxidase = 57.7%

mitochondrial contamination. The total recovery of cytochrome oxidase was 57.7%. The reason for this low recovery may be attributed to some loss of cytochrome oxidase in the supernatants SIII to SVI which had low protein content as compared to the SI and SII fractions. The results of cytochrome oxidase does, however, signify its marker enzyme activity.

(iii) Adenosine Deaminase:

Table V shows the determination and per cent distribution of adenosine deaminase in rat liver fractions obtained from the nuclear fractionation. Adenosine deaminase, which is exclusively a soluble phase enzyme, should only be found in the fractions containing the soluble phase. The results in Table V show that all the adenosine deaminase activity is recovered in SI and SII fractions which would contain the bulk of the soluble phase as expected. In all other fractions no adenosine deaminase activity was detected signifying the absence of soluble phase especially in the N fraction. The total recovery of adenosine deaminase is 76.2%. Some enzyme may have been inactivated in the supernatants containing low amounts of protein.

In determining the per cent distribution of total protein, NAD-pyrophosphorylase, cytochrome oxidase and adenosine deaminase, corrections were made for the amount of the homogenate (16 ml) removed for analysis.

Table V. Determination and Per Cent Distribution of Adenosine Deaminase in Rat Liver Fractions : Nuclei. The details of the assay can be found in the EXPERIMENTAL section of this chapter. Letting adenosine deaminase of the homogenate be 100 per cent, the percentages of adenosine deaminase in other fractions are shown. The total recovery should have been 100 per cent.

Table V

Determination and Per Cent Distribution of Adenosine Deaminase in Rat Liver Fractions : Nuclei

Fraction	Vol. in ml	Adenosine deaminase in units*	Per Cent
Homogenate	50	2.01	100
SI	80	1.39	69.2
SII	62	0.14	7.0
SIII	43	0	0
SIV	40	0	0
SV	40	0	0
SVI	34	0	0
N	15	0	0

*expressed in terms of micromoles of adenosine deaminated/min

Total recovery of adenosine deaminase = 76.2%

To summarize the results of the nuclear fractionation the nuclear fraction does not contain any PAH activity. The nuclei so obtained were of high purity as they contained all of the NAD-pyrophosphorylase activity, very little cytochrome oxidase activity which signifies extremely low mitochondrial contamination and no adenosine deaminase activity which indicates the absence of the soluble phase. The bulk of the PAH was retained in the SI fraction and other supernatants. These fractions were also pure as no nuclei were present in them. This was shown both by results of microscopic examination (Figure 3) and by the total lack of NAD-pyrophosphorylase activity. These fractions did, however, retain most of the cytochrome oxidase and adenosine deaminase activities as expected. (Tables IV and V). According to this fractionation, PAH is localized in one or more of the three cell components, mitochondria, microsomes and soluble phase.

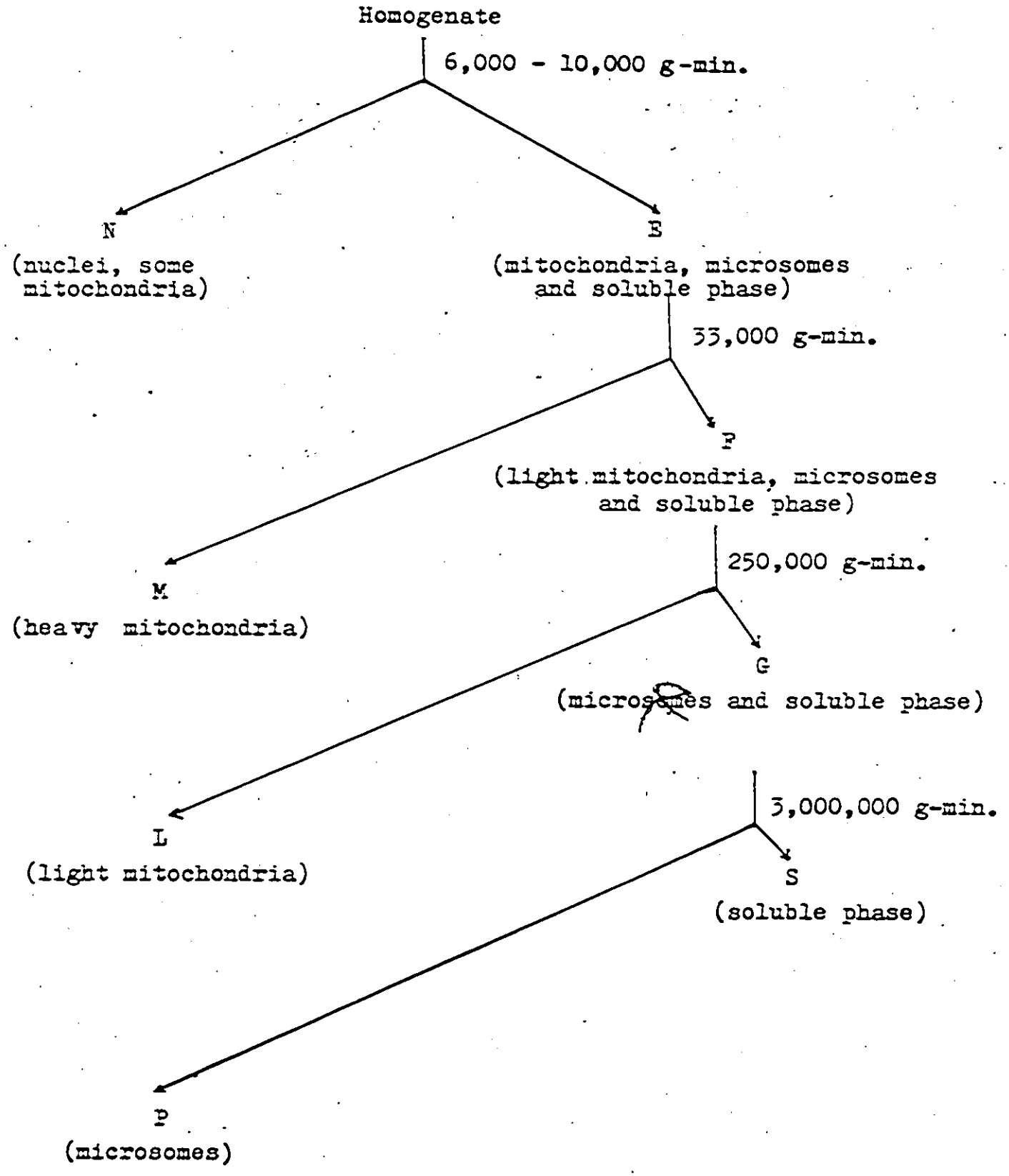
(b) Mitochondria, Microsomes and Soluble Phase

The mitochondria, microsomes and soluble phase were isolated and the flow sheet showing the steps of the fractionation is illustrated in Figure 5. Microscopic studies agreed with the implications shown in this flow sheet of the fractionation procedure. After centrifugations at 10,000 g-min. and 6,000 g-min., the N fraction was sedimented containing nuclei and some mitochondria. No

Figure 5. Flow Sheet showing Fractionation of Mitochondria, Microsomes and Soluble Phase of Rat Liver. The details of each step can be found in the EXPERIMENTAL section of this chapter. The components of each fraction are indicated.

Figure 5

Flow Sheet showing Fractionation of Mitochondria, Microsomes and Soluble Phase of Rat Liver.



whole cells, partially broken cells and cell debris were present in this fraction showing that homogenization of the tissue was complete. The supernatant, E contained the mitochondria, microsomes and the soluble phase. Centrifuging the E fraction at 33,000 g-min. sedimented the heavy mitochondria, the M fraction. This fraction was very dense and comprised of large mitochondria. The supernatant fraction F retained the light mitochondria, microsomes and soluble phase. Centrifuging the F fraction at 250,000 g-min. gave the L pellet which consisted of light mitochondria. The L fraction was less dense than the M fraction and was comprised of small mitochondria. Some heavy mitochondria were also observed. The supernatant, G contained the microsomes and the soluble phase. Mitochondria were not seen in this fraction.

The G fraction was centrifuged at 3,000,000 g-min. to give the pellet, P consisting of the microsomes and supernatant, S containing the soluble phase. The microsomes were barely visible under the magnification used. The fraction was fairly dense with negligible cell debris. The S fraction was clear with little cell debris. This fractionation procedure gave quite pure fractions of heavy and light mitochondria, microsomes and soluble phase as observed under the microscope.

Table VI shows the volumes, total protein, total PAH

Table VI. Enzyme PAH Localization in Rat Liver Fractions : Mitochondria, Microsomes and Soluble Phase. The details of the assay can be found in the EXPERIMENTAL section of this chapter. Protein was determined spectrophotometrically by the method of Warburg and Christian (44).

Table VI.

Enzyme FAH Localization in Rat Liver Fractions :

Mitochondria, Microsomes and Soluble Phase

Fraction		Volume ml	Total Protein mg	Total FAH Activity units*	Specific Activity units/mg x 100
H	N	40	1038	1.62	0.16
	E	100	2910	15.95	0.55
M		24	792	1.18	0.15
F		111	3230	14.70	0.46
L		26	647	0.34	0.05
G		120	1440	9.42	0.65
P		24	490	0.72	0.15
S		113	746	8.32	1.12

*Micromoles of propionopyruvate hydrolysed/min.

activity and specific activity of all the fractions for a single fractionation of rat liver. The homogenate, H represents the combined activity of nuclear fraction, N and cytoplasmic extract, E. After each split of a fraction by centrifugation the percentage of PAH activity recovered in the two resulting fractions was determined as illustrated in Figure 6 and the total recovery was calculated in which corrections were made for amounts of the fractions that were removed for analysis. This was done in fractions E, F and G from which 10, 4 and 5 ml were removed, respectively. Corrections were also made for fraction E in determining per cent distribution of PAH activity, total protein, cytochrome oxidase and adenosine deaminase activities. The average deviation from 100 per cent for PAH activity in Figure 6 was 12.7 per cent. About 33% of PAH activity was unaccounted for at a step of the light mitochondrial sedimentation. The reason for this will be discussed later.

Table VII shows the per cent distribution of PAH activity in rat liver fractions. The results from this table shows that the soluble phase fraction, S represents 47.4% of the PAH activity while the combined mitochondrial fractions, M and L shows 8.6% of the PAH activity which is over five times less than the S fraction. Also 4.1% and 9.2% of the PAH activity were shown in the microsomal pellet (P) and the nuclear fraction (N),

Figure 6. Balance Sheet for Total PAH Activity in Rat Liver Fractions : Mitochondria, Microsomes and Soluble Phase. After centrifugation two fractions arose from the initial fraction. The PAH activity of all three fractions was determined and the per cent recovery of PAH activity in the two resulting fractions was calculated. Corrections were made in the calculation of per cent recovery for the amount of the initial fraction that was removed for analysis and therefore the total recovery should have been 100 per cent. The actual total recovery is shown. The average deviation from 100% was 12.7 per cent.

Figure 6

Balance Sheet for Total PAH Activity in Rat Liver Fractions : Mitochondria, Microsomes and Soluble Phase

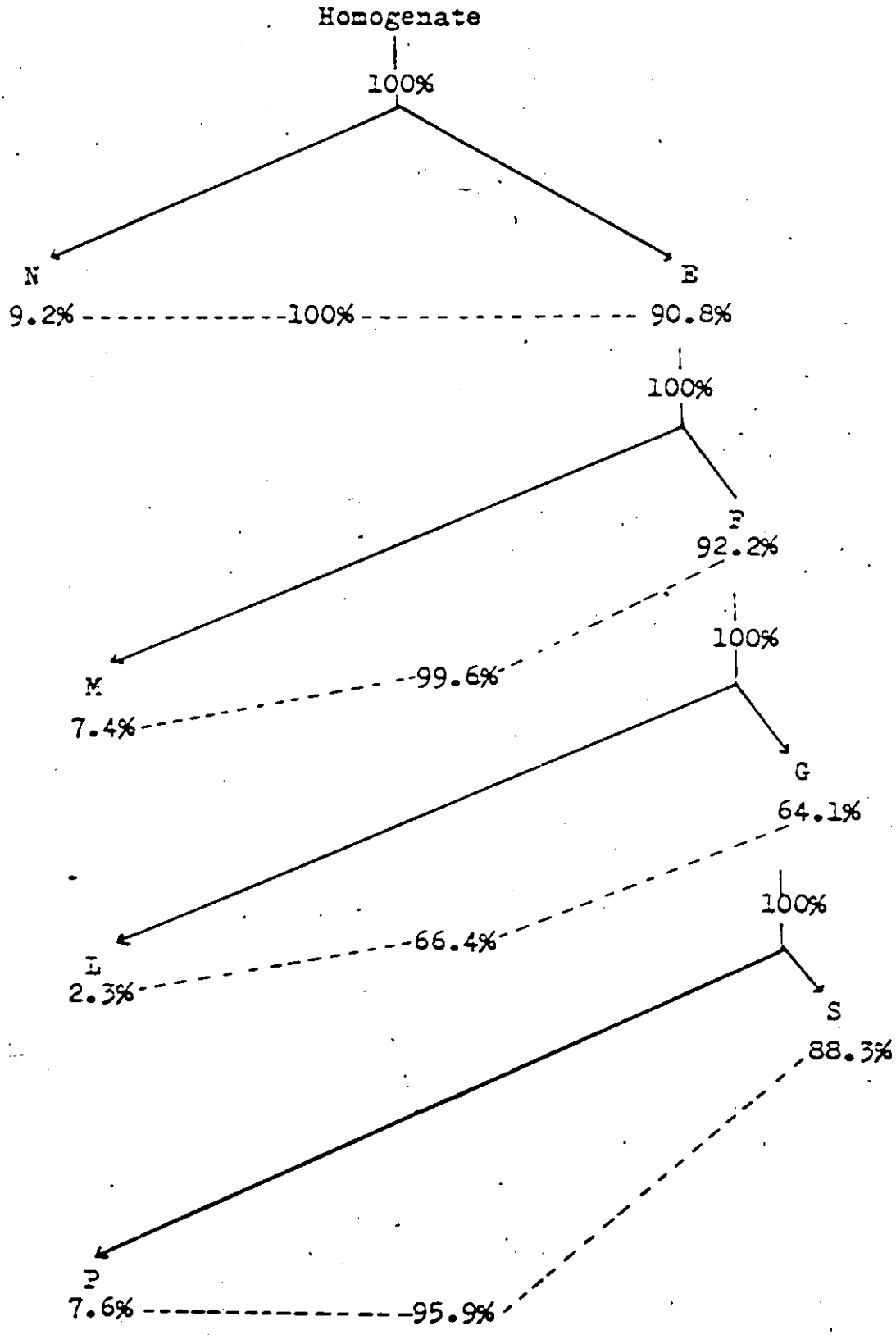


Table VII. Per Cent Distribution of PAH Activity and Total Protein in Rat Liver Fractions : Mitochondria, Microsomes and Soluble Phase. Letting both PAH activity and total protein in the homogenate, which is the sum of M and S, be 100 per cent, the percentages of PAH activities and total protein in five important fractions were calculated the total should be 100 per cent.

Table VII

Per Cent Distribution of PAH Activity and Total Protein in Rat Liver Fractions : Mitochondria, Microsomes and Soluble Phase

Fraction	PAH Activity %		Total Protein %	
N	9.2		26.3	
M	6.7	8.6	20.1	36.5
L	1.9		16.4	
P	4.1		12.4	
S	47.4		18.9	
Total	69.3		94.1	

respectively. Further deductions from these results are made after discussing the marker enzyme activities demonstrated in these fractions.

Table VII also shows per cent distribution of total protein in rat liver fractions obtained in mitochondrial fractionation. The results show that 56.5% of the protein is recovered in the M and L fractions combined. The S, N and P fractions retained 18.9, 26.3 and 12.4% of protein, respectively. The total recovery of protein is 94.1% with deviation from 100% of 5.9% which is within experimental error.

Marker Enzymes

(i) Cytochrome Oxidase.

The determination and per cent distribution of cytochrome oxidase in rat liver fractions obtained from the mitochondrial fractionation are shown in Table VIII. The results indicates that 61.4% of the cytochrome oxidase, mitochondrial marker enzyme, is recovered in the two mitochondrial fractions, M and L combined as expected. This shows that most of the mitochondira were sedimented in M and L fractions. However, 42.1% of cytochrome oxidase activity is found in the N fraction which means that the fraction is contaminated with some mitochondria as stated before in Figure 5. This contamination could have been minimized if the nuclear pellets were washed at least twice in small volumes of medium. This was not carried out since

Table VIII. Determination and Per Cent Distribution of Cytochrome Oxidase in Rat Liver Fractions : Mitochondria, Microsomes and Soluble Phase. The details of the assay can be found in the EXPERIMENTAL section of this chapter. In all cases 0.05 ml aliquots of enzyme fractions were used for assay except in case of fraction E where 0.1 ml aliquot was assayed. Letting cytochrome oxidase of the homogenate, which is M + E, be 100 per cent, the percentages of cytochrome oxidase in other fractions are shown. The total recovery should have been 100 per cent.

Table VIII

Determination and Per Cent Distribution of Cytochrome Oxidase in Rat Liver Fractions : Mitochondria, Microsomes and Soluble Phase

Fraction	Vol in ml	Cytochrome Oxidase in units*	Per Cent	
N + E (Homogenate)	40 + 100	2.4 + 3.3 (5.7)	100	
N	40	2.4	42.1	
M	24	2.0	35.1	61.4
L	26	1.5	26.3	
P	24	0.1	1.8	
S	113	0	0	

*$\Delta \log_{10}$[ferrochrome c] per min for 1 : 100 tissue dilution

Total recovery of cytochrome oxidase = 105.3%

the object of this fractionation was to prepare mitochondrial, microsomal and soluble phase fractions. The pellet, P showed 1.8% of the cytochrome oxidase activity which may mean that some very light mitochondria sedimented in this fraction. The supernatant, S did not show any cytochrome oxidase activity signifying that the fraction, S was free from mitochondrial contamination. The total recovery of cytochrome oxidase was 105.3% with deviation from 100% of 5.3% which is within experimental error.

(ii) Adenosine Deaminase:

The determination and per cent distribution of adenosine deaminase, a soluble phase marker, in rat liver fractions obtained from mitochondrial fractionation procedure are shown in Table IX. As expected, 72.2% of adenosine deaminase activity is retained in the soluble phase fraction, S.

The combined mitochondrial fractions M and L represents 19.4% of adenosine deaminase activity indicating that both M and L fractions had some soluble phase contamination. This may be because some soluble phase becomes associated with the membranes of the mitochondria in form of cytoplasmic tags which are very difficult to remove (65). Even after two washings many of them would still be associated with the mitochondria. To remove the cytoplasmic tags from the mitochondria, the mitochondrial

Table IX. Determination and Per Cent Distribution of Adenosine Deaminase in Rat Liver Fractions : Mitochondria, Microsomes and Soluble Phase. The details of the assay can be found in the EXPERIMENTAL section of this chapter. In all cases 0.05 ml aliquots of the enzyme fractions were assayed except in the case of fraction, S where 0.1 ml aliquot was assayed. Letting adenosine deaminase of the homogenate, which is E + N, be 100 per cent, the percentage of adenosine deaminase in other fractions are shown. The total recovery should have been 100 per cent.

Table IX

Determination and Per Cent Distribution of Adenosine Deaminase in Rat Liver Fractions : Mitochondria, Microsomes and Soluble Phase

Fraction	Vol in ml	Adenosine deaminase in units*	Per Cent	
N + E (Homogenate)	40 + 100	0.21 + 2.78 (2.99)	100	
N	40	0.21	7.0	
M	24	0.32	10.7	19.4%
L	26	0.26	8.7	
P	24	0.19	6.4	
S	113	2.16	72.2	

*expressed in terms of micromoles of adenosine deaminated/min

Total recovery of adenosine deaminase = 105.0%

pellet should be suspended gently in a volume of the medium in the centrifuge tube using Teflon homogenizer. The use of the centrifuge tube prevents mitochondrial disruption since the gap between the pestle and the tube is quite large. The mitochondria forms a fine suspension and the rotating pestle detaches the cytoplasmic tags from the mitochondria. This procedure may not remove the cytoplasmic tags completely. Also this treatment to the mitochondria may damage them for which reason, this procedure was not employed here. However, to remove the cytoplasmic tags completely, the outer mitochondrial membranes have to be removed (65). This principle cannot be used in case of the enzyme which is thought to be localized in the mitochondria especially the mitochondrial membranes.

The adenosine deaminase assay did show that most of the activity was found in the S fraction. The N fraction and the microsomal pellet, P showed low adenosine deaminase activities i.e. 7.0 and 6.4% respectively signifying little soluble phase contamination in these fractions. Total recovery of adenosine deaminase was 105% with deviation from 100% of 5% which is within experimental error.

(iii) Glucose-6-Phosphatase:

Table X shows the determination and per cent distribution of glucose-6-phosphatase (G6Pase), a

Table X. Determination and Per Cent Distribution of Glucose-6-Phosphatase in Rat Liver Fractions : Mitochondria, Microsomes and Soluble Phase. The details of the assay can be found in the EXPERIMENTAL section of this chapter. Letting glucose-6-phosphatase of the homogenate, which is M + S, be 100 per cent, the percentages of glucose-6-phosphatase in other fractions are shown. The total recovery should have been 100 per cent.

Table X

Determination and Per Cent Distribution of Glucose-6-Phosphatase in Rat Liver Fractions : Mitochondria Microsomes and Soluble Phase

Fraction	Vol in ml	Glucose-6-phosphatase in units*	Per Cent
N + E (Homogenate)	41 + 77 (118)	6.08 + 43.01 (49.09)	100
N	41	6.08	12.4
M	21	0.42	0.9
L	21	7.08	14.4
P	23.5	23.48	47.9
S	153	0	0

*expressed in terms of micromoles of P_i released/min

Total recovery of glucose-6-phosphatase = 75.6%

microsomal marker enzyme, in rat liver fractions obtained from second fractionation procedure. The majority of G6Pase activity (47.9%) was recovered in the microsomal pellet, P thus indicating that this fraction contained majority of the microsomes. On other hand no G6Pase activity was detected in the soluble fraction, S showing that this fraction was not contaminated by microsomes. The combined mitochondrial fractions retained 15.3% of G6Pase activity. This may be because some microsomes became closely associated with the mitochondria, mainly the light mitochondria, and this made very different to separate them in spite of repeated washings. However, G6Pase activity recovered in the microsomes is over three times than that recovered in combined mitochondrial fractions. The nuclear fraction, N shows 12.4% of the G6Pase activity. Total recovery of G6Pase was 75.6% which is quite reasonable because of the instability of the enzyme even at 0°C (66).

From the above results, the enzyme FAH is localized in the soluble phase fraction, S since it contained most of the cytoplasm as shown by 72.2% of adenosine deaminase activity (Table IX). The S fraction was free of mitochondria and microsomes as no cytochrome oxidase and G6Pase activities, respectively were detected in it (Table VIII and X). Thus the S fraction was obtained in a pure state and showed 47.4% of the FAH activity signifying that FAH is localized in the soluble phase.

The combined M and L fractions show 8.0% of the FAH activity. Most of the mitochondria are sedimented in these fractions as demonstrated by 61.4% of cytochrome oxidase activity being retained in them (Table VIII). However, combined M and L fractions show a fair amount of adenosine deaminase activity i.e. 19.4% signifying a fair amount of soluble phase contamination. Thus, it seems that 8.0% of the FAH activity present in these fractions, was due to the presence of some soluble phase in them.

The nuclear pellet, N and the microsomal pellet, P show 9.2 and 4.1% of FAH activities, respectively (Table VII). These activities in both N and P fractions seems to be due to soluble phase contamination as indicated by 7.0 and 6.4% of adenosine deaminase activities detected in these fractions respectively (Table IX).

The total recovery of FAH is 69.3% (Table VII). About 30% of FAH activity is unaccounted for. This may be due to some loss of FAH activity in the G supernatant at the stage when most of the protein (62.8%) was sedimented in N, M and L fractions (Table VII). The G fraction was very dilute i.e. a large volume of G (120 ml - Table VI) had relatively low protein content. Thus some amount of FAH may have split up into its subunits giving some loss of activity (67). Higher FAH activities in G as well as S were expected.

Table XI shows the distribution of FAH and total

Table XI. Distribution of PAH and Total Protein in Rat Liver : Soluble Phase, Microsomes, Mitochondria (heavy and light mitochondrial fractions are combined) and Nuclei. The data determined for the fractions containing each of these components is from the second fractionation procedure. The total PAH activity and total protein of rat liver tissue assigned to these four fractions are shown. The relative specific activity of each of these fractions has also been calculated where the relative specific activity is equal to the percentage of PAH activity in a fraction divided by the percentage of total protein in that fraction.

Table XI

Distribution of PAH and Total Protein in Rat Liver : Soluble Phase, Microsomes, Mitochondria and Nuclei

Fraction	% PAH Activity	% Total Protein	Relative specific Activity %PAH activity/% total protein
S	47.4	18.9	2.51
P	4.1	12.4	0.33
M + L	8.6	36.5	0.24
N	9.2	26.3	0.35
	TOTAL : 69.3	94.1	

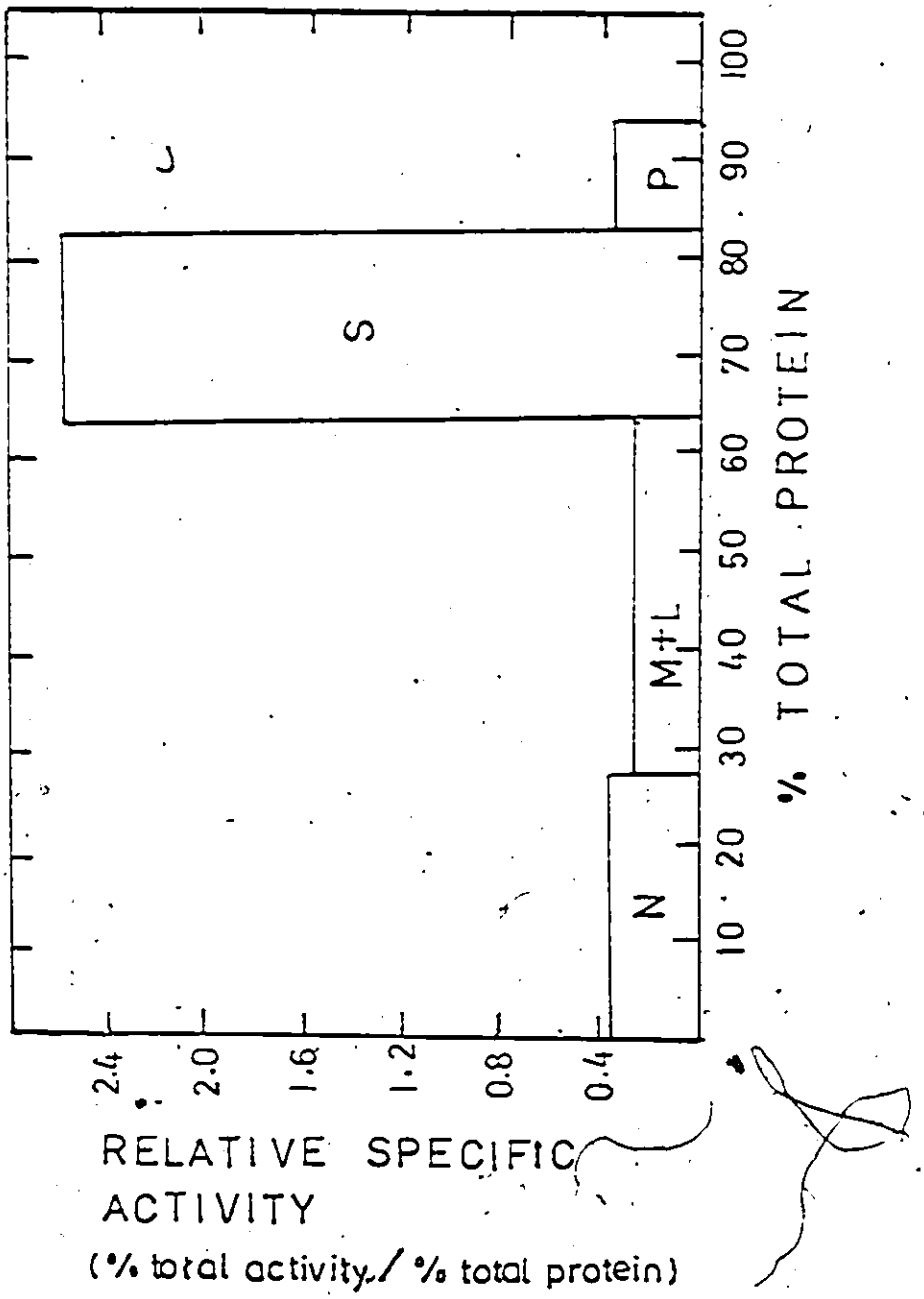
protein in rat liver soluble phase, microsomes, mitochondria and nuclei. The relative specific activities (RSA) of each of these fractions are also shown. The values calculated are from a single fractionation procedure. A distribution pattern of PAH in rat liver tissue is given in Figure 7. Results of Table XI shows that the RSA, expected as a ratio of the percentage of PAH to the percentage of total protein, for S fraction is 2.51 as compared to the RSA of 0.24 for M and L fractions combined.

RSA values of greater than 1.0 indicate that the enzyme is concentrated in the particular isolated fraction (45). For rat or mouse liver exclusive localization of an enzyme in a fraction would yield RSA values of approximately 10 for nuclei, 4 for mitochondria or microsomes and 3 for the soluble fraction (45). RSA of PAH in the S fraction obtained in this fractionation was 2.51, which is close to 3 as reviewed by Schneider (45), showing that PAH is exclusively localized in the soluble fraction of the rat liver. If more PAH was recovered in the S fraction, its RSA value would have been even higher. On the other hand RSA of the M and L fraction was only 0.24, which is over 10 times lower than the S fraction. This low value indicates that PAH is not localized in the mitochondria. Both P and N fractions also show low RSA values of 0.33 and 0.35, respectively indicating that PAH is not associated with these cell organelles.

To summarize the results of the mitochondrial,

Figure 7. Distribution Pattern of FAH in Rat Liver. The abcissa represents the percentage of the total protein contained in each fraction. The fractions are arranged in the following order : Nuclei, mitochondria, soluble phase and microsomes. The ordinate represents the relative specific activity of each fraction which equals the total activity in a fraction divided by the percentage of total protein in that fraction.

FIGURE 7


L
P
S
M+L
N
10 20 30 40 50 60 70 80 90 100
% TOTAL PROTEIN
2.4 2.0 1.6 1.2 0.8 0.4
RELATIVE SPECIFIC ACTIVITY
(% total activity / % total protein)

microsomal and soluble phase fractionation, the enzyme, PAH is localized in the S fraction. The S fraction so obtained contained most of the soluble phase (Table IX) and was not contaminated by mitochondria (Table VIII) and microsomes (Table X). The S fraction also has a high RSA value (1.51) for PAH (Table XI). Some PAH activity detected in the mitochondrial fractions, M and L seems to be due to contamination by soluble phase as shown by a fair amount of adenosine deaminase activity in these fractions (Table IX). These fractions also show very low RSA value for PAH.

CHAPTER III

DISCUSSION

Fumarylacetoacetate fumarylhydrolase or FAH catalyses the cleavage of fumarylacetoacetate into fumarate and acetoacetate. This reaction occurs in the final step of the degradative pathway of phenylalanine and tyrosine.

Rat liver tissue was chosen to determine the intracellular location of FAH. This tissue was fairly readily available and work could be done on fresh tissue. Also, studies of enzyme TAT of tyrosine catabolism was done on this tissue (19). Two separate fractionation procedures were adopted; one for obtaining pure nuclei and the other for obtaining mitochondrial, microsomal and soluble phase fractions.

The results of this study demonstrate that FAH is exclusively localized in the soluble phase of the rat liver. This was shown by 47.4% of FAH activity recovered in this fraction. Fractionation of mitochondria, microsomes and soluble phase show that the relative specific activity (RSA)of FAH in the S fraction was high i.e. 2.51, which is close to the RSA value of 3 to affirm exclusive localization in the soluble fraction (45). The combined mitochondrial fractions, M and L contain 8.6% of FAH activity. This activity seems to be due to some soluble phase contamination as shown by 19.4% of adenosine deaminase activity observed in these fractions.

Also the RSA of the combined M and L fractions was very low (0.24). These results show that PAH is not localized in the mitochondria of rat liver. The microsomal pellet, P represents 4.1% of the PAH activity and again this seems to be due to soluble phase contamination as indicated by 6.4% of adenosine deaminase activity found in this fraction. The nuclear fractionation shows that nuclei contained very low PAH activity i.e. 0.35 unit which is over 21 times less than PAH activity found in the SI fraction. This clearly indicates that PAH is not associated with the nuclei of the rat liver.

In the nuclear fractionation, the homogenate represents lower PAH activity and so the per cent distribution of PAH in the various fractions were not calculated. Per cent distribution of PAH activity in rat liver fractions obtained from the fractionation of mitochondria, microsomes and soluble phase, showed that the total recovery of PAH activity was 69.3%. About 30% of the PAH activity was unaccounted for based on the amounts recovered in the homogenate. This discrepancy may be due to some loss of PAH activity in G fraction at a stage when most of the protein was sedimented in the mitochondrial and the nuclear fractions leaving a relatively dilute G fraction. In this dilute fraction some of PAH may have split up into its subunits rendering it inactive (61).

Per cent distribution of total protein in the fractions obtained from nuclear fractionation showed that total

recovery was 106.7%. The total recovery of protein in rat liver fractions obtained from the fractionation of mitochondria, microsomes and soluble phase was 94.1%. Both recoveries were within experimental error.

There were two main ways in which the efficiency of the fractionation procedures were checked. The flow sheets of the fractionation represent the first method. Microscopic studies of each fraction confirmed the implications shown on these flow sheets. The components proposed to have been in each fraction after centrifugation were actually seen to have been present in each fraction.

Secondly, studies involving the distribution of marker enzymes were carried out to check the efficiency of fractionation procedures. The distribution of NAD-pyrophosphorylase, an exclusive nuclear enzyme showed convincingly that nuclei were present in the fraction where they were supposed to have been found. The results also show that NAD-pyrophosphorylase was absent in all other fractions signifying no contamination of nuclei in them. The total recovery of NAD-pyrophosphorylase was 82%. The distribution of cytochrome oxidase, a mitochondrial enzyme, showed that components of mitochondria were present in the proper fractions. Mitochondrial contamination of the nuclear fraction obtained from the first fractionation was extremely low since very low cytochrome oxidase was observed in it. The total recovery of cytochrome oxidase was low (57.7%). The reason for this

may be the result of some loss of cytochrome oxidase activity in dilute supernatant fractions which had relatively low protein content.

The third marker enzyme used was adenosine deaminase, an exclusive soluble phase enzyme. The results indicate that adenosine deaminase was found in fractions containing soluble phase as expected. The nuclear fraction did not show any adenosine deaminase activity indicating no soluble phase contamination in it. The total recovery of adenosine deaminase was 70.2%.

Per cent distribution of cytochrome oxidase in rat liver fractions obtained from the second fractionation showed that the majority of cytochrome oxidase activity was found in the combined mitochondrial fractions as expected. The microsomal pellet showed very low cytochrome oxidase activity signifying very low mitochondrial contamination while the soluble phase did not show any cytochrome oxidase activity indicating an absence of mitochondria in this fraction. Per cent distribution of adenosine deaminase in the same fractions showed that most of the activity was recovered in the S fraction as expected signifying the fraction retained the bulk of the soluble phase. Some adenosine deaminase activity was also found in the combined mitochondrial fraction indicating some soluble phase contamination in them.

A microsomal marker enzyme, G6Pase was also used to

check the efficiency of mitochondrial, microsomal and soluble phase fractionation. As expected, the results show that the majority of G6Pase was found in microsomal pellet. The soluble phase did not show any G6Pase activity indicating that soluble phase was not contaminated with microsomes. The nuclear and the combined mitochondrial pellets showed some G6Pase activities. The total recovery of G6Pase was 79.6%.

In brief, results of the marker enzymes indicate that all important fractions from both fractionation procedure were obtained in pure states.

For the second fractionation procedure, efficiency was also checked by determining PAH activity in each step of centrifugation and total recovery of the two resulting fractions was calculated. In fractions S and L, the total recovery was low, the reason for which was discussed earlier. Recoveries at other steps were close to 100%.

Thus the enzyme PAH is localized in the soluble phase of the rat liver. The indirect evidence of Williams and Sreenivasan (24) supports this finding. They showed that the complete tyrosine oxidising system is found in the 25,000x g supernatant and none in the precipitate, which included most of the microsomes. Also PAH was first purified and separated from the soluble fraction of rat liver (37). The findings here demonstrate that the end of phenylalanine and tyrosine degradation is located in the soluble fraction of the rat liver cell. The enzyme, maleylacetoacetate isomerase involved in the step

before FAH in tyrosine metabolism was also purified and separated from the soluble fraction of rat liver (37). The enzyme homogentisic acid oxidase was also found in the soluble fraction of liver and kidney (35). It can be speculated that the last three steps of the phenylalanine and tyrosine catabolism occur in the soluble fraction of rat liver. However, exclusive localization of maleyl-acetoacetate isomerase and homogentisic acid oxidase should be done before this can be said with any certainty.

FAH may be speculated to exist in one form in liver tissue. Mahuran et al. (68) determined the molecular weight of FAH from beef liver by polyacrylamide disc gel electrophoresis and found only one band. They also carried out isoelectrofocussing of FAH from beef liver over a range of pH 3.5 to 10 at 300v and showed one peak with the pI (isoelectric pH) of 6.7 . However, similar studies should be redone on rat liver tissue and isoelectrofocussing should be performed over a narrow pH range (5.5 to 7.5) before existence of a single form of FAH could be established.

CHAPTER IV

SUMMARY

The intracellular location of FAH has been demonstrated in rat liver tissue. Two fractionation procedures involving homogenization and differential centrifugation were adopted. One method yields the nuclear fraction while the other method yields the soluble phase, microsomal and heavy and light mitochondrial fractions. Few modifications were made for the nuclear fractionation.

The nuclear fractionation showed that the nuclear fraction retained only 0.35 unit of FAH activity and 2.1% of total protein while the SI fraction, containing majority of the mitochondria, microsomes and soluble phase, retained 7.56 units of FAH activity and 64.0% of the total protein. The other supernatant SII to SVI retained between 0.27 to 0.5 unit of FAH activity and between 0.1 to 30% of total protein.

The results of the second fractionation showed that based on the amount of FAH activity and total protein content of the homogenate: 47.4% of FAH activity and 18.9% of the total protein was contained in the soluble phase; 8.6% of FAH activity and 36.5% of total protein was contained in the combined mitochondrial fractions; the microsomal fraction contained only 4.1% of FAH activity and 12.4% of the total protein. The relative specific activities (% FAH activity / % total protein) of soluble phase, combined mitochondrial, microsomal and nuclear fractions were 2.51, 0.24, 0.33, 0.35, respectively.

The efficiency of fractionation was checked by two methods in both fractionations. Microscopy studies revealed that each fraction contained only its proper components. The second method for fractionation efficiency involved the analyses for the presence of the marker enzymes in the fractions. An exclusive nuclear enzyme, NAD-pyrophosphorylase, an exclusive mitochondrial enzyme, cytochrome oxidase, an exclusive soluble phase enzyme, adenosine deaminase and a microsomal marker enzyme, glucose-6-phosphatase were utilized and majority of their activities were restricted to their proper fractions as expected i.e. 82% of NAD-pyrophosphorylase was retained in the nuclear fraction; 53.3% of cytochrome oxidase was recovered in the combined SI and SII (mitochondrial, microsomal and soluble phase) fractions obtained from the nuclear fractionation and 61.4% of cytochrome oxidase was recovered from the combined M and L (mitochondrial) fractions obtained from the second fractionation; 76.2% of adenosine deaminase activity was retained in the soluble phase fractions from nuclear fractionation and 72.2% of adenosine deaminase retained in the soluble phase fraction obtained from the second fractionation; 47.9% of G6Pase activity was recovered from the microsomal fraction. However, the combined mitochondrial fractions of the second fractionation contained 19.4% of adenosine deaminase activity indicating some soluble phase contamination.

In the second fractionation, efficiency was also checked

by per cent recovery of FAH activity in the two fractions resulting from the centrifugation of a fraction for every step in the fractionation.

The enzyme FAH is exclusively localized in the soluble phase of the rat liver cell. Some FAH activity found in the mitochondrial fractions and microsomal fraction was probably due to the low levels of soluble phase contamination in them.

APPENDIX

The Nomenclature of g-min.

Briefly g-min. on Sorvall RC2-B centrifuge, SS-34 head were calculated as follows :

$$\text{g-min.} = \text{gav} \times (\text{time of centrifugation in min})$$

where g-min. ≡ composite unit of the force prevailing in the middle of the fluid of the centrifuge tube

$$\text{gav} = \frac{1}{981} w^2 \text{Rav}$$

where 981 ≡ g in cm/sec^2

$$\text{Rav} = (R_{min} + R_{max})/2$$

where R_{min} and R_{max} are the radii measured from the centres of the tube to the axis of rotation (cm).

w^2 = angular momentum (rad/sec)

$$w = \frac{2\pi}{60} v \quad (v \equiv \text{velocity in rpm})$$

$$\text{gav} = \frac{1}{981} \left(\frac{2\pi}{60}\right)^2 v^2 \text{Rav}$$

$$= 0.817 \times 10^{-4} v^2$$

for the Sorvall SS-34 cent. head; Rav has been calculated to be 7.28 cm

For International Preparative Ultracentrifuge Model B-60, A170 centrifuge head:

R_{min} = 5.0 cm

R_{max} = 8.5 cm

R_{av} = 6.75 cm

$$gav = \frac{1}{981} \left(\frac{2}{60}\right)^2 v^2 Rav = 0.749 \times 10^{-4} v^2.$$

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